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Systematic review and new insights into the molecular characterization of the *Candida rugosa* species complex ☆

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ABSTRACT

Recently, *Candida rugosa* was characterized as a species complex comprising four taxa: *C. rugosa sensu stricto*, *Candida pseudorugosa*, *Candida neorugosa* and *Candida mesorugosa*. Although considered relatively rare, several clusters of candidemia due to *C. rugosa* complex had been reported presenting mortality rates close to 70%. In this work we discuss the systematization, phenotyping and molecular methods based on internal transcribed spacer region (ITS) sequencing and proteomic analyses for species identification, as well as clinical aspects of the *C. rugosa* complex. We performed a Bayesian phylogenetic analysis using 72 ITS sequences representative of *C. rugosa* complex isolates and related species within the genus. Biochemical, morphological and MALDI-TOF MS analyses were processed with *C. rugosa* complex type strains and related species isolates. We described that the phylogeny showed four distinct clades inferred with high posterior probabilities, corresponding to the four species within the *C. rugosa* complex, excluding *C. pararugosa*. Biochemical and morphological aspects distinguished only *C. rugosa sensu stricto* but were not sufficient to accurately identify species within the rest of the complex. Protein spectrum profiles differentiated all reference strains from different species analyzed. To our knowledge, we presented the first phylogenetic analysis using a large collection of ITS sequences as well as proteomic profiles generated from isolates of the *C. rugosa* complex and related species that can enlighten systematics, diagnostics and clinical research fields.

1. Introduction

Candida rugosa is an ascomycetous yeast organism that appears to be emerging as an etiological agent of human infectious diseases in different parts of the world (Pfaller et al., 2006). This species is most frequently found in environmental sources and is a cause of bovine mastitis, which is one of the most important diseases in dairy cows (Crawshaw et al., 2005; Scaccabarozzi et al., 2011). However, in recent years, *C. rugosa* has been described as a cause of candidemia in critically ill trauma patients (Behera et al., 2010; Pfaller et al., 2006; Singh et al., 2011), with the isolation frequency estimated at 0.6% worldwide, but a higher prevalence in South America (2.7%) (Pfaller et al., 2006).

Candida rugosa is phenotypically characterized by the formation of macroscopic wrinkled colonies that vary in color from white to cream and microscopic blastoconidia and pseudohyphae (De Hoog et al., 2000). This species is an anamorph yeast without a described sexual cycle (Calderone and Clancy, 2012). It has been extensively studied as a source of extracellular lipases that can be used in the production of several compounds, such as fatty acids and esters (Benjamin and Pandey, 1998; Dominguez de Maria et al., 2006).

Historically, *C. rugosa* was first named *Mycoderma rugosa* by Anderson after isolation from human feces in 1917 (Moretti et al., 2000). It possessed species synonymies in the botanical field for decades, known as *Azymocandida rugosa*, *Mycotorula rugosa* and *Torula rugosa*. Finally, in 1942, *C. rugosa* was reclassified by Diddens and Lodder (Meyer et al., 1998).

In recent years, with the knowledge gained from molecular studies, systematic and taxonomic classifications of molds and yeasts have been dramatically revised. *Candida rugosa* is now addressed as a complex of different species that encompasses *C. rugosa*, *C. pseudorugosa*, *C. neorugosa* and *C. mesorugosa* (Chaves et al., 2013; Paredes et al., 2012).

Due to the novelty of the molecular characterization of cryptic species within the *C. rugosa* complex, there is a lack of robust and consistent data on the putative clinical peculiarities and antifungal susceptibility of all four species. Consequently, most of the publications on human infections caused by strains of the *C. rugosa* complex had not provided accurate identification of species by gene sequencing (Colombo et al., 2003; Minces et al., 2009).

In the three largest series of candidemia due to the *C. rugosa* complex, crude mortality rates ranged from 44% to 68% (Behera et al., 2010; Colombo et al., 2003; Dube et al., 1994). It remains

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unclear whether high mortality rates reported by patients with hematogenous infections are mostly related to the severity of illness and comorbidities present at the time of the diagnosis of fungemia or whether this finding may be related to the limited antifungal susceptibility of *C. rugosa* to amphotericin B and fluconazole (Behera et al., 2010; Colombo et al., 2003).

In addition to hematogenous candidiasis, yeasts of the *C. rugosa* complex have been rarely isolated from the respiratory tract, urine, feces, skin and peritonitis (Kocyigit et al., 2010; Li et al., 2006; Paredes et al., 2012; Tay et al., 2011).

Authors had little success in demonstrating that *C. rugosa* isolates produce virulence factors, *e.g.*, secreted enzymes. Among different species of *Candida* spp. tested, *C. rugosa* isolates were negative for phospholipase and protease activities (Kantarcioglu and Yucel, 2002), and could only produce alpha hemolysis (Luo et al., 2001). Lipase secretion by *C. rugosa* strains has been extensively investigated, and these enzymes have many applications in biotechnological processes (Benjamin and Pandey, 1998; Dominguez de Maria et al., 2006). However, it remains unclear whether *C. rugosa* secreted lipases have any relevance to colonization or infection in human hosts, suggesting that *C. rugosa* complex is one of the less virulent of the genus.

The focus of the present work is to review taxonomic and diagnostic aspects of the *C. rugosa sensu lato* providing new data related to proteomic profiles as well as a phylogenetic approach to enlighten the systematics of the species within this complex.

2. Material and methods

2.1. Phylogenetic analysis

To perform the phylogenetic analysis, ITS sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) after BLASTn searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the sequences from type strains of each species within the *C. rugosa* complex and *C. pararugosa* as queries: *C. rugosa* CBS 613^T or ATCC 10571^T (AY500374), *C. mesorugosa* CBS 12656^T (FJ768909), *C. pseudorugosa* CBS 10433^T (DQ234792), *C. neorugosa* CBS 12627^T (HE716762) and *C. pararugosa* ATCC 38774^T (AF421856). Only sequences that presented identity and coverage of $\geqslant$80% with query sequences with low levels of gaps and/or ambiguities were considered in the analysis, to include a more representative sample of interspecies and intraspecies variations (Ciardo et al., 2006; Nilsson et al., 2008). Additionally, the ITS sequences of *C. albicans* (FJ662406), *C. dubliniensis* (AB369916), *C. parapsilosis* (EU564205), *C. metapsilosis* (EU484054) and *C. orthopsilosis* (EU557373), that represent well-known species complexes were added for comparison. All sequences were aligned using the muscle algorithm implemented by SEAVIEW 4.2.12 (Gouy et al., 2010) and adjusted by eye before phylogenetic analysis. An unconstrained consensus phylogeny was inferred with MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with default priors as input. The number of generations was 2.5 million, where the average standard deviation of split frequencies was <0.01, with data saved every 100 generations and run in four chains and two runs. During the runs, the GTR model and shape of the gamma distribution parameters, as well as the proportions of invariant sites, were estimated.

2.2. Phenotypic and biochemical characterization of the C. rugosa species complex and related species

The colony morphology of *C. rugosa* ATCC 10571^T, *C. mesorugosa* CBS 12656^T, *C. pseudorugosa* CBS 10433^T, *C. neorugosa* CBS 12627^T, and *C. rugosa* (*pararugosa*) CBS 1948^T was determined on Sabouraud Dextrose Agar (SDA) (BD Difco, USA) plates after 48 h of incubation at 35 °C. CHROMagar Candida (BD, USA) plates were incubated at 35 °C for 72 h before colony color observation. For micromorphological observation, cells were cultured in Yeast Peptone Dextrose (YPD) broth and incubated overnight at 30 °C and also in cornmeal agar plates incubated at 35 °C for 72 h. For biochemical and assimilation analyses, ID32C (BioMerieux, France) was used according to the manufacturer's instructions.

2.3. Mass spectrometry analysis of the Candida rugosa complex and related species

All tested *Candida* sp. isolates were recovered from frozen stock onto SDA and incubated for 3 days at 35 °C before experiments. To obtain the proteins from the isolates, we first tested the protocol described by Stevenson et al. (2010), but we found that *C. rugosa* ATCC 10571^T, which formed very wrinkled colonies, and *C. rugosa* (*C. pararugosa*) CBS 1948^T did not yield reproducible results. Therefore, we adapted the protocol by growing the isolates in YPD broth overnight at 30 °C before proceeding with the analysis. One milliliter of cultured cells was pelleted and washed twice with ultrapure water, and the excess of water was removed. A small loop of cells was directly spotted onto each position of a Micro Scout Plate (MSP) 96 polished steel target (Bruker Daltonics GmbH, Germany). Then, 25% formic acid was immediately poured onto the samples. After drying at room temperature, each sample was overlaid with matrix solution, which consisted of a saturated solution of alpha-cyano-4-hydroxy-cinnamic acid (Sigma, USA) in 50% acetonitrile/2.5% trifluoroacetic acid (Sigma, USA), and the matrix/sample was co-crystallized by air-drying at room temperature. Each isolate was spotted in triplicate. Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, BrukerDaltonics). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.5 kV; mass range, 2000–20,000 Da). For each spectrum, 100 shots in 50-shot steps from different positions of the target spot (automatic mode) were collected and analyzed. Spectra were internally calibrated by using *Escherichia coli* ribosomal proteins. MALDI Biotyper software 3.0 (Bruker Daltonik GmbH) was used to visualize mass spectra and generate the MSP dendrogram.

3. Results and discussion

3.1. Taxonomy, systematics and geographic trends of different species within the C. rugosa complex

In the past years, the high genetic heterogeneity of the *C. rugosa* taxon has gained attention (Dib et al., 1996; Redkar et al., 1996; Scaccabarozzi et al., 2011; Tay et al., 2011), and more studies on the morphology, biochemical patterns and sequencing analysis, mostly based on ribosomal RNA genes, have impacted the taxonomy of *C. rugosa*.

The novel species within the *C. rugosa* complex have been identified based on 26S D1/D2 rDNA sequences, complemented with ITS sequences (Butinar et al., 2011; Chaves et al., 2013; Li et al., 2006; Paredes et al., 2012; Taverna et al., 2012; Tay et al., 2011). Of note, only *C. mesorugosa* was characterized based on sequences of rDNA regions as well as housekeeping genes (Chaves et al., 2013).

To our knowledge, no complete genome of any species within the *C. rugosa* complex is available. Chaves et al. (2013) succeeded in amplifying fragments of *ACT1* and *COX2* of *C. rugosa*, *C. mesorugosa* and *C. pseudorugosa*, but the *RBP1* gene fragment could not be amplified from one of the *C. pseudorugosa*-like isolates, and none of

the three housekeeping genes were amplified from *C. rugosa* (*C. pararugosa*) CBS 1948^T (Chaves et al., 2013).

The fungal barcoding initiative has recently proposed the use of ITS sequences to accurate identify fungal species (Schoch et al., 2012). Therefore, analysis of the ITS region may not only suffice for those who want rapid species identification by sequence comparisons with type strains, but also, be applied to infer evolutionary relationships among cryptic and newly characterized taxa.

Here, we describe the phylogenetic relationships based on a comprehensive analysis of ITS sequences obtained from different strains representative of all species within the *C. rugosa* complex and related species (Table 1 and Fig. 1). The *C. rugosa* species complex includes four distinct clades, inferred with high posterior probabilities (pp), that corresponded to *C. rugosa sensu stricto* and three recently described species: *C. pseudorugosa*, *C. neorugosa* and *C. mesorugosa* (Fig. 1) (Chaves et al., 2013; Paredes et al., 2012). Despite *C. pseudorugosa* and *C. neorugosa* split had occurred earlier than *C. rugosa sensu stricto* and *C. mesorugosa*, the first two species were considered as part of the *C. rugosa* complex because they still can be misidentified as *C. rugosa* when only phenotypic and biochemical characteristics are analyzed.

In our analysis, the *C. rugosa sensu stricto* clade was divided into two distinct groups with a pp of 0.78: group I accounted for strains mostly isolated from human or animal sources in the United States, United Kingdom and Thailand and grouped with *C. rugosa* ATCC10571^T type strain (CBS 613^T and WM234), whereas in group II, the majority of the isolates were from environmental sources, originally from China and India (Table 1). Tay and collaborators (2011) have described two *C. rugosa* subgroups when analyzing sequences of rDNA: subgroup A, related to environmental isolates, and subgroup B, related to clinical isolates (Tay et al., 2011). However, they observed by BLAST analysis that both ITS and 26S D1/D2 sequences of *C. rugosa* subgroup B shared greater identity with sequences from *C. rugosa* ATCC 10571^T than subgroup A (93.7 and 99.6% *versus* 89.2 and 99.1, respectively). Indeed, in our analysis, the ITS sequence from isolate STC4 (subgroup B) grouped with *C. rugosa* environmental isolates (*C. rugosa* group II), whereas the isolate STC1, which belongs to their subgroup A, clustered within the clade of the novel species *C. mesorugosa* (Chaves et al., 2013). Therefore, STC1 should be acknowledged as a representative of the *C. mesorugosa* taxon.

Candida pseudorugosa was described in 2006 (Li et al., 2006), and it was first isolated from a sputum sample of a Chinese patient who died of respiratory failure, septic shock, and deterioration. After the original report, two *C. pseudorugosa*-like isolates recovered in 2001 from subglacial ice in the coastal Arctic environment of Kongsfjorden, Spitzbergen, were reported (Butinar et al., 2011). The species identification was verified with sequencing analysis of the 26S D1/D2 region, and the identity and coverage between the two environmental isolates and the *C. pseudorugosa* type strain sequences were 99% and 100%, respectively. Finally, Taverna et al. (2012) recently described the isolation of specimens from blood and central venous catheters of a female patient in Argentina that were closely related to *C. pseudorugosa* (Taverna et al., 2012). In our analysis (Fig. 1), the *C. pseudorugosa* clade appeared diverse, with several uncharacterized isolates grouping with the reference strain and with well-identified *C. pseudorugosa* isolates. The sequences from those uncharacterized isolates might be representatives of different subspecies, varieties or even novel species within the *C. pseudorugosa* clade. However, this hypothesis remains to be confirmed by further analysis of other genes and by increasing the number of isolates tested to ensure consistency in this clade.

Most recently, two novel *C. rugosa*-related species were described: *C. neorugosa* and *C. mesorugosa* (Chaves et al., 2013; Paredes et al., 2012). *Candida neorugosa* is closest to the *C. pseudorugosa* clade and has only three characterized isolates related to clinical sources, two isolated in the USA and one in Brazil. *Candida mesorugosa* is another diverse group. In our phylogenetic analysis, we discriminated two groups: the first one grouped sequences from strains isolated from animal and environmental sources in Pakistan and India; the second group encompassed strains isolated from elephant feces from Pakistan with the *C. mesorugosa* type strain (CBS 12656^T) and other well-characterized clinical isolates from Brazil. The two groups within the *C. mesorugosa* clade were separated with *pp* = 0.63, suggesting that the intraspecific variability of the ITS sequences analyzed was low. Therefore, other DNA markers with faster substitution rates as mitochondrial genes would be useful to address the phylogenetic relationships of the isolates within the *C. mesorugosa* taxon.

Although the *C. pararugosa* taxon is not part of the *C. rugosa* species complex according to molecular analysis, misidentification of this species as *C. rugosa* is common when only phenotypic characteristics are analyzed. Fig. 1 shows that *C. pararugosa* is a distinct clade, with *pp* = 1. Isolates from different sources and countries can be found, such as isolates obtained from foods (cheeses and koumiss) in Spain, Canada and China, and isolates obtained from clinical sources, mostly from the USA and one isolate from Brazil (Table 1).

3.2. Limitations of phenotypic tests for accurate identification of species within the C. rugosa complex and related taxa

Although molecular methods are considered the gold standard for yeast identification, morphological and biochemical tests are still used by most clinical laboratories for *Candida* sp. identification. Below, we present data on morphological aspects of the colonies and biochemical tests that may be useful in a first screening for species identification of the *C. rugosa* species complex.

3.2.1. Morphological aspects

In our study *C. rugosa* ATCC 10571^T presented white, wrinkled and fringed colonies on SDA plates (Fig. 2), in accordance with a previous description by (Meyer et al., 1998). In contrast, *C. pseudorugosa* CBS 10433^T, *C. neorugosa* CBS 12627^T and *C. mesorugosa* CBS 12656^T showed white, smooth colonies, whereas *C. pararugosa* CBS 1948^T presented smaller and flat colony.

By culturing the reference strains on CHROMagar Candida, each tested species generated different colony colors: dry light blue for *C. rugosa*, blue-pink for *C. mesorugosa,* dark blue for *C. pseudorugosa*, pale pink for *C. pararugosa* and dark blue-violet for *C. neorugosa.*

In terms of micromorphology, the *C. rugosa* isolate exhibited abundant pseudohyphae in both YPD broth and cornmeal agar. Production of pseudohyphae was incipient in *C. pseudorugosa* and absent in *C. mesorugosa*, *C. neorugosa* and *C. pararugosa* grown in YPD broth. On cornmeal agar plates, those four species presented few and/or rudimentary pseudohyphae and many blastoconidia. Taken together the findings considering the type strains, we may suggest that macro and micromorphological aspects, such as, wrinkled and dry aspect of the colony with abundant pseudohyphae, have a potential for discriminating *C. rugosa sensu stricto* from other closely related species. However, more studies using a larger collection of strains within the *C. rugosa* complex should be performed to better support our observations.

3.2.2. Biochemical tests

Candida rugosa is a non-fermentative yeast, so biochemical assimilation tests are essential to differentiate this species from other *Candida* spp. (Meyer et al., 1998). As described by Meyer et al. (1998), *C. rugosa* isolates can assimilate glucose (+), galactose (+), L-sorbose (v), D-xylose (v), N-acetyl-D-glucosamine (late +), ethanol (+) glycerol (+), ribitol (−/late +), D-mannitol (+/late +),

Table 1
Strains and sequences obtained from BLAST comparisons of the *C. rugosa* species complex analyzed in the present study.

Strain	Source/isolation site	Country	ITS GenBank no.	References
C. rugosa ATCC10571[T], or CBS 613[T], or WM234	Human feces	–	GU144663 AY500374 EF568037	Mota et al. (unpublished), Pryce et al. (2003), Kong et al. (unpublished)
C. rugosa UTHSC 06-3729	Human ear	USA	HE716760	Paredes et al. (2012)
C. rugosa CBS 2016	Dung of scouring cow	UK	GU246265	Groenewald and Smith (2010)
C. rugosa NRRL 95	–	USA[a]	AY533550 and AY533551[b]	Ren and Chaturvedi. (unpublished)
C. rugosa Z-HS5	Soil	Pakistan[a]	JF896570	Latif and Amin. (unpublished)
C. rugosa Z-HS13	Soil	Pakistan[a]	JF896571	Latif and Amin. (unpublished).
Candida sp. 10-1	Elephant feces	Thailand	AB727605	Lorliam, et al. (unpublished).
C. rugosa STC4 subgroup B	Human blood	Malaysia	HM641832	Tay et al. (2011)
C. rugosa Zhuan8	Marine yeast	China[a]	EF197805	Guo and Chi. (unpublished)
C. rugosa strain 8	Marine yeast	China[a]	EF198009	Liu and Chi. (unpublished)
C. rugosa strain UY	Contaminated soil	India	JQ974952	Dandi et al. (unpublished)
C. rugosa UTHSC R-3412	Human	Spain[a]	HE716759	Paredes et al. (2012)
C. rugosa STC1 subgroup A	Human blood	Malaysia	HM641831	Tay et al. (2011)
C. mesorugosa L69D (CBS12656)[T]	Human blood	Brazil	FJ768909	Chaves et al. (2013)
C. mesorugosa L154	Human blood	Brazil	FJ768910	Chaves et al. (2013)
C. mesorugosa L387A	Human rectal swab	Brazil	FJ768911	Chaves et al. (2013)
C. mesorugosa L412D	Human pericatheter swab	Brazil	FJ768912	Chaves et al. (2013)
C. mesorugosa L2683B	Human blood	Brazil	FJ768913	Chaves et al. (2013)
C. mesorugosa UOA/HCPF2	Human blood	Greece	GQ376074	Meyer et al. (unpublished)
Candida sp. 2-1	Elephant feces	Thailand	AB727593	Lorliam et al. (unpublished)
Candida sp. 3-2	Elephant feces	Thailand	AB727594	Lorliam et al. (unpublished)
Candida sp. 5-1	Elephant feces	Thailand	AB727595	Lorliam et al. (unpublished)
Candida sp. 5-2	Elephant feces	Thailand	AB727598	Lorliam et al. (unpublished)
Candida sp. 5-3	Elephant feces	Thailand	AB727597	Lorliam et al. (unpublished)
Candida sp. 7-1	Elephant feces	Thailand	AB727599	Lorliam et al. (unpublished)
Candida sp. 7-8	Elephant feces	Thailand	AB727600	Lorliam et al. (unpublished)
Candida sp. 9-1	Elephant feces	Thailand	AB727601	Lorliam et al. (unpublished)
Candida sp. 9-2	Elephant feces	Thailand	AB727602	Lorliam et al. (unpublished)
Candida sp. 9-3	Elephant feces	Thailand	AB727603	Lorliam et al. (unpublished)
Candida sp. 9-4	Elephant feces	Thailand	AB727604	Lorliam et al. (unpublished)
Candida sp. 10-2	Elephant feces	Thailand	AB727606	Lorliam et al. (unpublished)
Candida sp. 10-4	Elephant feces	Thailand	AB727608	Lorliam et al. (unpublished)
Candida sp. 10-5	Elephant feces	Thailand	AB727609	Lorliam et al. (unpublished)
Candida sp. 10-8	Elephant feces	Thailand	AB727610	Lorliam et al. (unpublished)
Candida sp. VITGBN1	Industrial wastewater	India	KC135883	Basak and Das (unpublished)
Candida sp. VITJzN04	Agricultural soil	India	JX454449	Salam and Das (unpublished)
Candida sp. D15	Insect	Bulgaria	HM627153	Gouliamova et al. (unpublished)
Candida sp. D24	Insect	Bulgaria	HM627160	Gouliamova et al. (unpublished)
Candida sp. Envir-L230	Environmental	Brazil	JX245057	Chaves et al. (2013)
Candida sp. Envir-L231	Environmental	Brazil	JX245058	Chaves et al. (2013)
Candida sp. DMic 103837	Catheter	Argentina	JF345209	Taverna et al. (2012)
Candida sp. DMic 103838	Catheter	Argentina	JF345210	Taverna et al. (2012)
Candida sp. DMic 103839 (CBS 12267)[T]	Human blood	Argentina	JF345211	Taverna et al. (2012)
Candida sp. DMic 103840	Human blood	Argentina	JF345212	Taverna et al. (2012)
Candida sp. DMic 103841	Human blood	Argentina	JF345213	Taverna et al. (2012)
Candida sp. DMic 103842	Human blood	Argentina	JF345214	Taverna et al. (2012)
C. pseudorugosa XH1164 (CBS 10433)[T]	Human sputum	China	DQ234792	Li et al. (2006)
C. pseudorugosa UTHSC 06-3641	Human catheter urine	USA	HE716755	Paredes et al. (2012)
C. pseudorugosa UTHSC 08-707	Human knee	USA	HE716756	Paredes et al. (2012)
C. neorugosa UTHSC 10-2054 (CBS12627)[T]	Human leg wound	USA	HE716762	Paredes et al. (2012)
C. neorugosa UTHSC 10-121	Horse leg wound	USA	HE716761	Paredes et al. (2012)
Candida sp. SK75	Human ulcerated lesion	Brazil[a]	GQ176145	Mota et al. (unpublished)
C. rugosa (*C. pararugosa*) CBS1948[T]	Human sputum	Norway	JX245060	Chaves et al. (2013)
C. pararugosa UTHSC 08-442	Human urine	USA	HE716757	Paredes et al. (2012)
C. pararugosa UTHSC 09-2953	Human vagina	USA	HE716758	Paredes et al. (2012)
C. pararugosa UWFP-348	Human	USA	AF335925 and AF335965[b]	Chen et al. (2001)
C. pararugosa KKA Seq 1226	Human	Germany	EF519702	Forster et al. (unpublished)
C. pararugosa ATCC 38774[T]	Human feces	–	AF421856	Chen et al. (2001)
C. pararugosa MA09-AP	Cheese	Canada	GQ458032	Arteau et al. (2010)
C. pararugosa IDR1000011225	–	USA[a]	JN675331	Chaturvedi and Chaturvedi. (unpublished)
C. pararugosa "MOTA"	Human oral cavity	Brazil[a]	GQ139517	Mota et al. (unpublished)
C. pararugosa M 0166	Koumiss	China	EU780147	Chen. (unpublished)
C. pararugosa 3AD19	Cheese	Spain	DQ646685	Alvarez-Martin et al. (2007)
C. pararugosa ZIM 2433	Cheese	Serbia	HE799663	Cadez et al. (unpublished)
C. pararugosa ZIM 2438	Cheese	Serbia	HE799664	Cadez et al. (unpublished)

Table 1 (*continued*)

Strain	Source/isolation site	Country	ITS GenBank no.	References
C. parapsilosis L6492	Human blood	Brazil	EU564205	Gonçalves et al. (unpublished)
C. metapsilosis L7685	Human blood	Brazil	EU484054	Gonçalves et al. (unpublished)
C. orthopsilosis L7956	Human blood	Brazil	EU557373	Gonçalves et al. (unpublished)
C. dubliniensis IFM53163	Human sputum	Japan	AB369916	Sano et al. (unpublished)
C. albicans ZA046	Human oral	China	FJ662406	Zheng et al. (unpublished)

[T] Type strain.
[a] Country assigned by the location of the university or research center from which the sequence of the isolate was generated.
[b] ITS1-5.8S and 5.8S-ITS2 sequences merged into a single contig to be used in the phylogenetic analysis.

D-glucitol (+/late +), D-gluconate (v), DL-lactate (+/late +), succinate (v), citrate (v), and hexadecane (late +).

The accuracy of phenotypic methods in the identification of species within the *C. rugosa* complex has been evaluated in new studies comprising a limited number of strains. Overall, databases of all commercial systems include only the *C. rugosa* taxon. By checking the identification of *C. rugosa* strains with API 20C, ID 32C and Vitek YBC, the rates of inconsistent results and/or misidentification ranged from 42% to 100% compared to DNA sequencing (Cendejas-Bueno et al., 2010; Chaves et al., 2013; Ciardo et al., 2006; Paredes et al., 2012).

Recently, Paredes et al. (2012) suggested a panel of four assimilation tests that could discriminate *C. rugosa*, *C. pseudorugosa* and *C. neorugosa* (Table 2). It is noteworthy that in that particular study, only two strains representative of each species were tested, and no single type strain was incorporated into the analysis. Moreover, these data disagree with a previous publication by Meyer et al. (1998) and the results found in our laboratory with the same assimilation tests using the type strains (Table 2). In conclusion, the validation of the Paredes and collaborators strategy for screening species within the *C. rugosa* complex should be confirmed by further studies enrolling a larger number of strains.

3.3. Proteomics for species identification within the C. rugosa complex

Together with DNA profiling, protein fingerprinting has been used to identify the genus and species of relevant microorganisms in industrial and medical scenarios (Fox, 2006). Protein fingerprinting relies on matrix-assisted laser-desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS), in which proteins within a complex mixture are analyzed in less than 2 min. Mass spectra of the most abundant proteins of the isolate in culture range from 1000 to 20,000 Da. The final genus or species identification is provided by comparisons of peaks from fingerprints deposited in a curated library with peaks of the spectrum generated from the sample (Bruker and Daltonics, 2008).

The libraries widely used to identify species by MALDI-TOF technology, *e.g.*, BioTyper (Bruker Daltonics, Bremen, Germany) and SARAMIS (bioMérieux, Potsdam-Golm, Germany), contain the spectra of *C. rugosa* and *C. pararugosa*, but none of them is up to date with the novel taxonomic classification of the *C. rugosa* species complex (Bruker and Daltonics, 2008; Saramis, 2009).

Marklein et al. (2009) tested the accuracy of MALDI-TOF MS identification compared with conventional tests. They tested 285 isolates from medically important yeasts, and 92.5% (247) of the isolates were accurately identified by proteomics. The remaining isolates were later identified after complementation of the database with protein spectra of the missing type strains. The single *C. rugosa* isolate tested in the paper was accurately identified after the database complementation was completed (Marklein et al., 2009).

In 2010, Stevenson and collaborators created a spectral database library using the MALDI BioTyper2 software (Bruker Daltonics) for 109 type strains. Afterwards, 194 clinical isolates belonging to 23 species of six genera were tested. They reported an identification accuracy of 99.0% (192 isolates), including six *C. rugosa* isolates with spectral score $\geqslant 1.8$. The two clinical isolates that were not identified were one *C. rugosa* and one *Cryptococcus neoformans*, which may reflect the heterogeneity of isolates within the *C. rugosa* species complex (Stevenson et al., 2010).

In the present study, we report the profiles generated by MALDI-TOF MS analysis of six reference strains and four clinical isolates representative of the *C. rugosa* species complex. The most representative spectrum profiles that differentiated the species of the isolates presented peaks ranging from 2000 to 10,000 Da, with two regions with the most intense peaks at 3000–4000 and 6000–8000 Da (Fig. 3). Fig. 3 depicts the dendrogram that successfully discriminated all type strains from the different species tested and grouped the 5 isolates belonging to the novel *C. mesorugosa* species. Despite we used a few isolates, proteomic data corroborate our phylogenetic analysis using ITS sequences and also confirm the intraspecies variations of *C. mesorugosa* strains isolated from different regions of Brazil, as previously observed by our group using RAPD and multilocus analyses (Chaves et al., 2013).

Taking the above results together, we conclude the molecular identification of the *C. rugosa* species complex at the species level may be achieved by using MALDI-TOF MS and ITS region sequencing analyses.

3.4. Considerations on the clinical aspects related to C. rugosa complex

Despite being considered a rare human pathogen, there are several reports of cluster episodes of fungemia due to *C. rugosa* documented in patients admitted to intensive care units. According to Minces et al. (2009), a total of 27 episodes of candidemia were reported in the English literature since its first description in 1985, including an outbreak of six cases in Brazil and a large series of invasive candidiasis documented in burn patients in California (Colombo et al., 2003; Dube et al., 1994; Minces et al., 2009; Reinhardt et al., 1985; Sugar and Stevens, 1985). Recently, Behera et al. (2010) reported an additional cluster of fungemia due to *C. rugosa* in 19 critically ill trauma patients in a teaching hospital of north India.

Considering the epidemiological data provided by the three largest series of candidemia mentioned above, we may suggest that the main conditions associated with episodes of *C. rugosa* fungemia are tegument disruption by invasive medical procedures or burns as well as previous exposure to nystatin, parenteral nutrition and broad-spectrum antibiotics (Behera et al., 2010; Colombo et al., 2003; Dube et al., 1994). Of note, a substantial number of cases have been documented in patients older than 60 years, as noted by Tay et al. (2011) in their report of two cases of fungemia (Tay et al., 2011).

Indeed, there is much controversy about the *in vitro* susceptibility of strains of the *C. rugosa* complex to antifungal drugs. Pfaller et al. (2006) found that only 40% of 462 *C. rugosa* strains tested

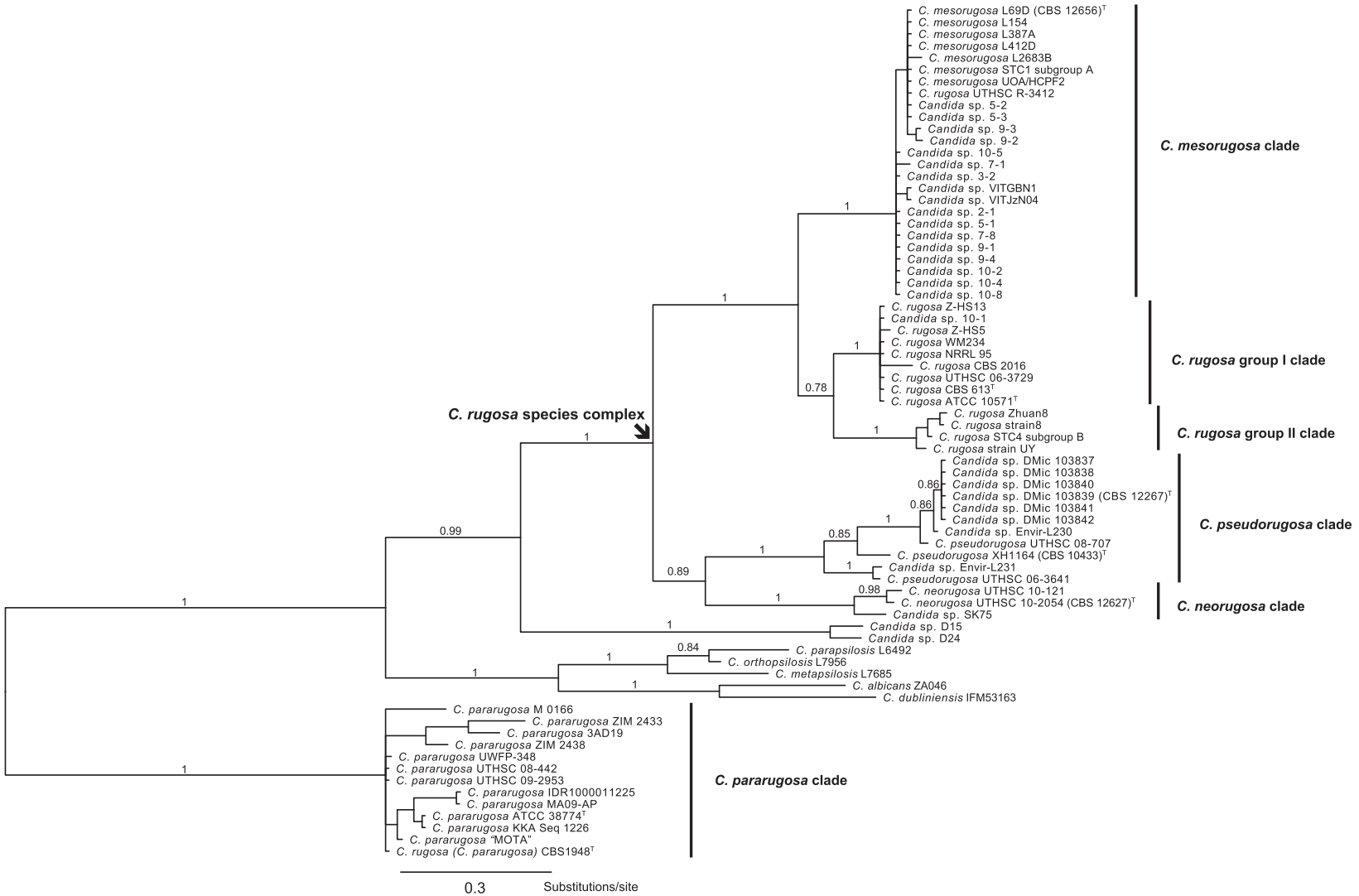

Fig. 1. Unrooted Bayesian consensus phylogenetic tree of the *Candida rugosa* species complex and other *Candida* spp. based on confidently aligned ITS rDNA sequences (Table 1). Posterior probabilities above 0.75 are depicted in the main nodes. The Bayesian tree was inferred from 2.5 million generations with burn-in = 6250. Runs were saved every 100 generations and run in four chains and two runs. The selected model was $fA = 0.296757$, $fC = 0.216826$, $fG = 0.206238$, and $fT = 0.280179$, with rate matrix [A–C] = 0.133394, [A–G] = 0.225850, [A–T] = 0.156289, [C–G] = 0.111998, [C–T] = 0.265260 and [G–T] = 0.107209. The shape parameter of the gamma distribution was $\alpha = 0.389278$, and the proportion of invariant sites was $I = 0.019057$.

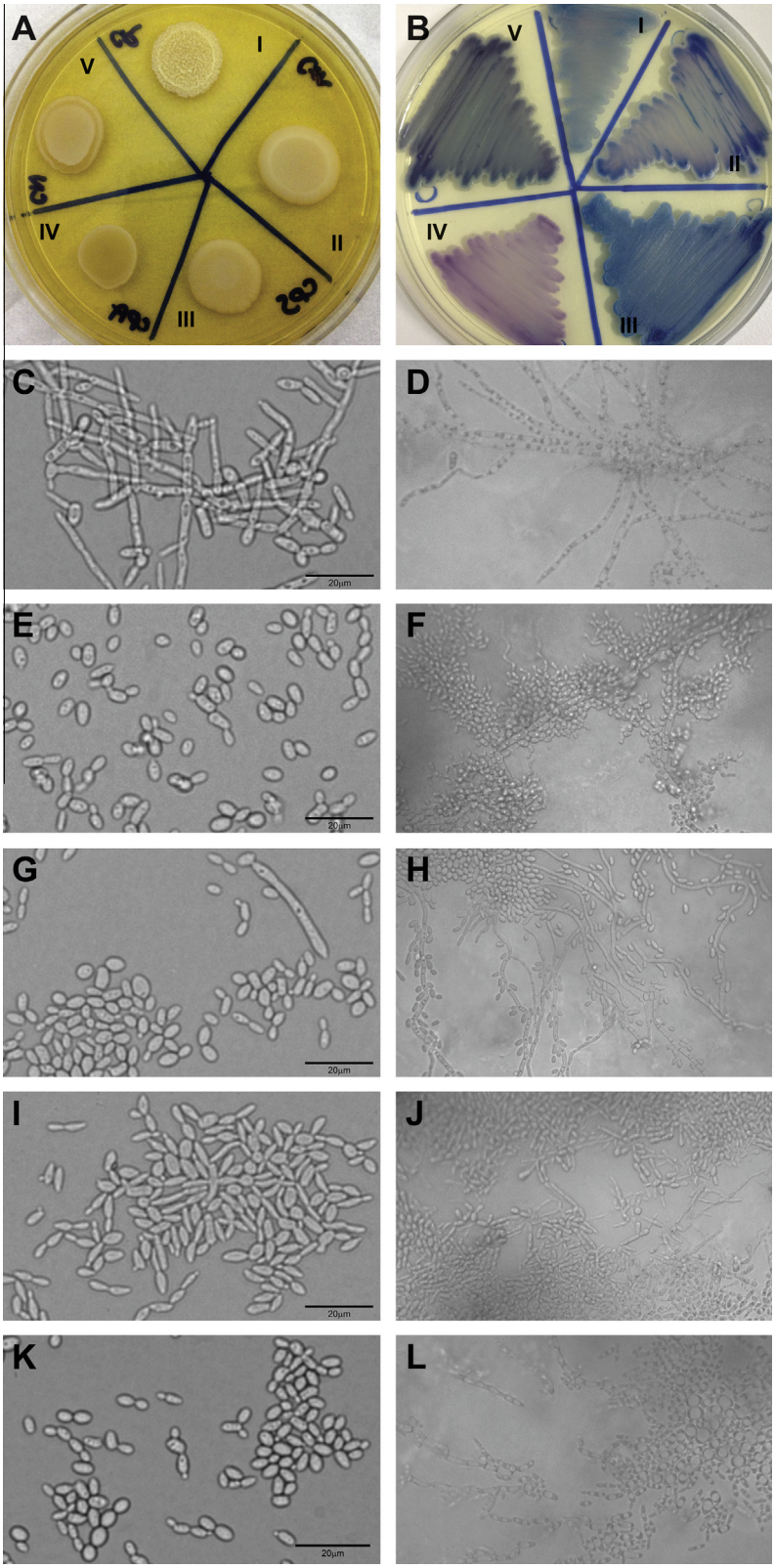

Fig. 2. (**A** and **B)** Macromorphology of colonies from (I) *C. rugosa* ATCC 10571^T, (II) *C. mesorugosa* CBS 12656^T, (III) *C. pseudorugosa* CBS 10433^T, (IV) *C. rugosa* (*C. pararugosa*) CBS 1948^T and (V) *C. neorugosa* CBS 12627^T type strains grown on SDA and CHROMagar *Candida*, respectively. Micromorphology of cells grown in YPD broth (left column) and on cornmeal agar (right column), with 400X magnification. Letters identify respectively: (**C** and **D)** *C. rugosa* ATCC 10571^T; (**E** and **F)** *C. mesorugosa* CBS 12656^T; (**G** and **H)** *C. pseudorugosa* CBS 10433^T; (**I** and **J)** *C. rugosa (C. pararugosa)* CBS 1948^T; and (**K** and **L)** *C. neorugosa* CBS 12627^T.

exhibited low minimal inhibitory concentrations (MIC's) to fluconazole. In a previous series reported by our group, three of six patients were infected by strains exhibiting high MIC's against fluconazole (Colombo et al., 2003). Behera et al. (2010) tested 19 strains and found only 20% of them with high MIC's to fluconazole, though all them exhibited low MIC's to amphotericin B and voric-

Table 2
Assimilation tests proposed for identification of the *C. rugosa* species complex. Results obtained by different authors.

	Authors								
	Paredes et al. (2012)				Present study[a]				Meyer et al. (1998)
Species	*C. rug*	*C. neo*	*C. pse*	*C. mes*	*C. rug*	*C. neo*	*C. pse*	*C. mes*	*C. rugosa sensu lato*
Carbon sources									
SOR	+	+	–	NT	+	+	+	+	v
XYL	+	+	–	NT	+	+	+	+	v
RIB	–	+	–	NT	–	+	–	–	–
GLY	+	+	–	NT	+	+	–	+	+

[a] The present study tested only type strain isolates from each species *C. rug*: *C. rugosa*; *C. neo*: *C. neorugosa*; *C. pse*: *C. pseudorugosa*; *C. mes*: *C. mesorugosa* SOR: Sorbose; XYL: D-xylose; RIB: ribitol; GLY: glycerol +: positive; –: negative; v: variable; NT: Not tested.

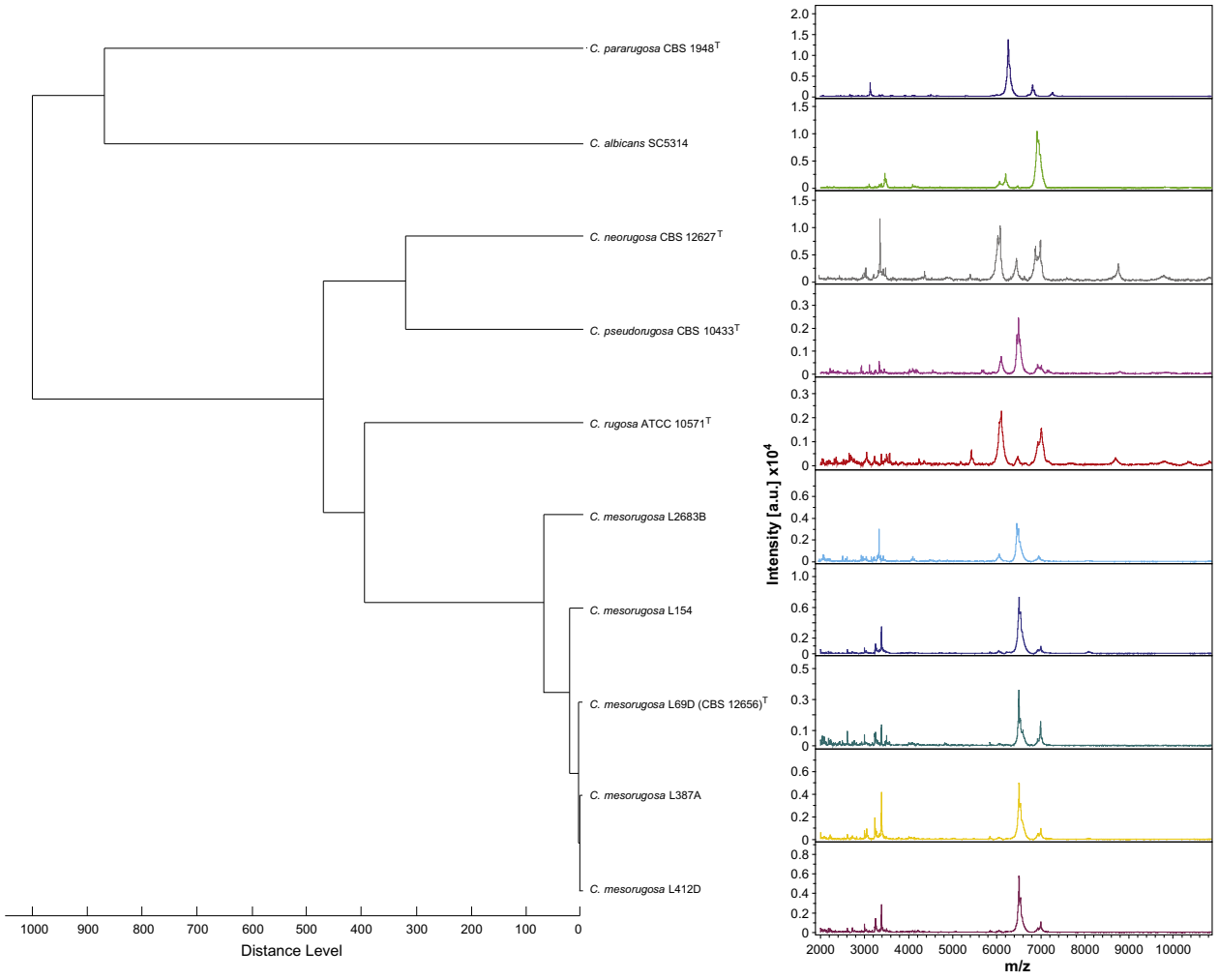

Fig. 3. Dendrogram of the main spectra of the *C. rugosa* species complex and *C. albicans* and *C. pararugosa* reference strains. Distance values are relative and normalized to a maximum value of 1000. In detail, representative spectra from the isolates analyzed in this study. *m*/*z*: mass to charge ratio.

onazole. Paredes et al. (2012) tested 10 strains of *C. rugosa sensu stricto* and found that all presented low MIC's to triazoles, amphotericin B and candins.

Finally, based on the large discrepancies in the results described by different studies, it is possible that there is a high variability of antifungal MIC's within strains of the *C. rugosa* complex that may be related to geographic variations as well as inter- and intraspecies variations. Consequently, based on the uncertainty of the best therapeutic choice for treating invasive infections of *C. rugosa*, decisions on antifungal therapy should be made case by case, considering the accurate species identification, clinical status of the patient, the site of infection and the *in vitro* antifungal susceptibility of the particular isolate.

4. Conclusions

The accurate identification of the four species within the *C. rugosa* complex and *C. pararugosa* is highly dependent on molecular methods. Here we showed that ITS sequencing analysis appears to be the most reliable for identification and proteomics

has a potential to discriminate species within the *C. rugosa* complex. Our phylogenetic analysis using ITS sequences were adequated for phylogenetic and evolutionary analyses, which consolidated the four taxa within the *C. rugosa* complex as distinct species and also demonstrated high diversity among the isolates within each clade, which may reflect an ongoing diversification process. Finally, we propose that collaborative and multicentric studies enrolling medical centers from different geographic areas are needed to investigate the putative biological differences and clinical features of infections caused by each species of the *C. rugosa* complex, as well as to validate new, reliable diagnostic tools and therapeutic strategies.

Declaration of interest

Dr. Colombo has received research and educational grants from Astellas, MSD, Pfizer and United Medical in the last two years. All the other authors report no conflicts of interest relevant to this article. The authors alone are responsible for the content and the writing of the paper.

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